Institut für Veterinärpathologie
der Vetsuisse-Fakultät Universität Zürich

Direktorin: Prof. Dr. Anja Kipar

Arbeit unter wissenschaftlicher Betreuung von
Prof. Dr. Anja Kipar

Co-Infecting Reptarenavirus Species Can Be Vertically Transmitted in *Boa Constrictor*

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Saskia Keller

Tierärztin
von Kloten, ZH

genehmigt auf Antrag von

Prof. Dr. Anja Kipar, Referentin
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Koinfizierende Reptarenaviren können in *Boa constrictors* vertikal übertragen werden.

Boid inclusion body disease (BIBD) ist eine meist tödlich verlaufende Erkrankung bei Boas und Pythons. Seit Kurzem werden Infektionen mit *Reptarenaviren* (Familie *Arenaviridae*) mit BIBD in Zusammenhang gebracht und als Ursache vermutet, wobei Einzel- und Koinfektionen mit diversen Virusspezies vorkommen. In der vorliegenden Arbeit konnte erstmals die vertikale Übertragung einer Reptarenavirus-Infektion und/oder Koinfektion nachgewiesen werden. Fünf Würfe mit Nachkommen verschiedener Altersgruppen (Embryonen, perinatale Aborte und Juvenile) wurden auf BIBD und Reptarenaviren untersucht. Die Mutter- und/oder Vatertiere waren entweder histologisch positiv für die pathognomonischen Einschlusskörperchen (EK) oder für Reptarenavirus-RNA im Gewebe. Mittels "Next Generation Sequencing" und nachfolgender "*de novo* Assembly" konnten nahezu vollständige Genome mehrerer Spezies von Reptarenaviren in den einzelnen Würfen nachgewiesen und miteinander verglichen werden. Virusspezies-spezifische RT-PCRs bestätigten die vertikale Übertragung der Ko-Infektionen. Auch in Zellkulturen aus embryonalem Gewebe erfolgte eine Virusreplikation und EK entwickelten sich schnell. Virusantigen war bereits im Gewebe von Embryonen und perinatalen Aborten nachweisbar, jedoch fanden sich EK konstant erst in juvenilen Tieren ab einem Alter von zwei Monaten, was darauf hindeutet, dass Arenavirusinfektionen über einen längeren Zeitraum zu BIBD führen.

Stichwörter: BIBD (Boid inclusion body disease), Reptarenaviren, Koinfektion, vertikale Übertragung

Vetsuisse Faculty University of Zurich 2016

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Co-Infecting Reptarenavirus Species Can Be Vertically Transmitted in *Boa Constrictor*

Boid inclusion body disease (BIBD) is an often fatal disease affecting mainly constrictor snakes. BIBD has been associated with infection, and more recently with co-infection, by various species of the genus *Reptarenavirus* (family *Arenaviridae*). Thus far neither the incubation period nor the route of transmission of BIBD are known. Herein we demonstrate that co-infecting reptarenavirus species can be vertically transmitted in *Boa constrictor*. We examined five *Boa constrictor* clutches with offspring ranging in age from embryos over perinatal abortions to juveniles. The mother and/or father of3 each clutch were initially diagnosed with BIBD and/or reptarenavirus infection by detection of the pathognomonic inclusion bodies (IB) and/or repatrenaviral RNA. Using next-generation sequencing and *de novo* sequence assembly we sequenced the "arenavirome" of each clutch, yielding several nearly complete genomes of multiple reptarenvavirus species. We further confirmed vertical transmission of the co-infecting reptarenaviruses by species-specific RT-PCRs applied to samples from parental animals and offspring. Also, cell cultures derived from embryonal samples rapidly developed IB and promoted replication of some or all parental viruses. Viral antigen was detecte in some embryos and perinatal abortions, but IB were consistently seen only in the juvenile snakes from the age of 2 mo onwards, indicating that reptarenavirus infection induces BIBD over time in the offspring.

Keywords: BIBD (Boid Inclusion Body) Disease, Reptarenavirus, vertical transmission, Co-infection

Co-Infecting Reptarenavirus Species Can Be Vertically Transmitted in Boa Constrictor

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ABSTRACT

Boid inclusion body disease (BIBD) is an often fatal disease affecting mainly constrictor snakes. BIBD has been associated with infection, and more recently with coinfection, by various species of the genus *Reptarenavirus* (family *Arenaviridae*). Thus far BIBD has only been reported in captive snakes, and neither the incubation period nor the route of transmission are known. Herein we demonstrate that co-infecting reptarenavirus species can be vertically transmitted in boa constrictor. In total we examined five boa constrictor clutches with offspring ranging in age from embryos over perinatal abortions to juveniles. The mother and/or father of each clutch were initially diagnosed with BIBD and/or reptarenavirus infection by detection of the pathognomonic inclusion bodies (IB) and/or reptarenaviral RNA. By applying next-generation sequencing and *de novo* sequence assembly we sequenced the "arenavirome" of each clutch, yielding several nearly complete genomes of multiple reptarenavirus species. We further confirmed vertical transmission of the co-infecting reptarenaviruses by species-specific RT-PCR from samples of parental animals and offspring. Curiously, not all offspring obtained the full parental "virome". We further supported our findings by an *in vitro* approach; cell cultures derived from embryonal samples rapidly developed IB and promoted replication of some or all parental viruses. In the tissues of embryos and perinatal abortions, viral antigen was sometimes detected, but IB were consistently seen only in the juvenile snakes from the age of 2 mo onwards. In addition to demonstrating vertical transmission of multiple species, our results also indicate that reptarenavirus infection induces BIBD over time in the offspring.

INTRODUCTION

Boid inclusion body disease (BIBD) is a transmissible, progressive and generally fatal disease of boid snakes. First described in the 1970s, BIBD subsequently emerged as a major problem in boid snake collections worldwide [1, 2]. Several genera of boid species have been reported as susceptible to the disease, but its prevalence among snakes as well as its potential occurrence in wild populations is yet unknown [3]. Clinically, BIBD is highly variable particularly in boas, where affected animals can be free of clinical signs, die from secondary infections, or develop neurological signs. The latter are generally more pronounced in pythons. The hallmark of BIBD are the characteristic intracytoplasmic electron dense inclusion bodies (IB) that are found in most cell types [1, 2, 4, 5]. The pathogenesis of BIBD is not yet characterized, and both subclinical as well as chronic disease has been described [2, 6].

A few years ago a novel group of arenaviruses were identified in and isolated from snakes with BIBD [4, 5, 7]. Arenaviruses are negative-sense RNA viruses with two genome segments, L and S, which encode Z protein and RNA-dependent RNA polymerase, and glycoprotein precursor and nucleoprotein (NP), respectively [8]. Strong evidence of the causative relationship between reptarenavirus infection and BIBD is provided by the ability of reptarenavirus isolates to induce the pathognomonic IB in an *in vitro* model [4], and by the fact that the IB contain or mainly consist of reptarenavirus NP [4, 5, 9]. The identification of BIBD-associated arenaviruses led to the formation of a new genus, *Reptarenavirus*, in the family *Arenaviridae*, placing the previously known arenaviruses to another new genus, *Mammarenavirus* [8]. More recently, we and others observed that snakes with BIBD are often co-infected with different reptarenavirus species; in one snake, for example, four distinct S and 11 distinct L reptarenavirus segments were found [10, 11]. The genomes of reptarenaviruses are highly variable [4, 5, 7, 10-13], as a consequence, the diagnosis of BIBD still relies mainly on the detection of IB in cells in tissues or in blood smears by light microscopy, complemented with immunohistochemistry.

So far, the route of transmission and the incubation period of reptarenaviruses are unknown, and direct contact or vector mediated transmission by snake mites (*Ophionyssus natricis*) have been proposed [1, 14]. In line with the "transmission through a vector" hypothesis, we recently reported the growth of reptarenaviruses also in arthropod cell lines [14]. In addition, vertical transmission from dam to offspring in both egg-lying and live-bearing snakes has been considered by Chang and Jacobson [1], but so far this hypothesis has not been investigated. Furthermore, studies on the vertical transmission in reptiles are scarce and include only few viruses, such as equine encephalitis virus [15], adenovirus [16]; Herpesvirus M [17, 18] and, very recently, Sunshinevirus [19]. Mammarenaviruses can be vertically transmitted in their reservoir rodent hosts [20-23]. Prenatal infection plays an important role in virus maintenance, and, at least in the case of Lymphocytic choriomeningitis virus (LCMV), Machupo virus (MACV) and Lassa virus (LASV), leads to chronic infection [24].

We set up this study to determine whether reptarenaviruses can be vertically transmitted. For this purpose, five *Boa constrictor* clutches, represented by parental animals diagnosed with BIBD by traditional methods, or RT-PCR positive for reptarenavirus, and their offspring, ranging from embryos in the first trimester to 12-month-old juveniles, were examined. We applied next-generation sequencing (NGS) to characterise the "arenavirome" of each clutch, and used virus-specific RT-PCRs to confirm the findings. Primary cell cultures originating from the embryos served to evaluate the potential of the infecting viruses to induce IB formation and thereby also the disease.

RESULTS

BIBD and reptarenavirus infection in the parents and offspring

The diagnosis of BIBD is confirmed when the characteristic cytoplasmic IB are seen within cells. These IB contain abundant reptarenavirus NP which can be visualised by immunohistology [4, 5, 9]; RT-PCR can serve to confirm reptarenavirus infection. We verified the parental animals as BIBD positive and/or positive for reptarenavirus infection using histology and immunohistology. For clutches 1 and 3-5 this was complemented by a robust RT-PCR targeting a subset of known reptarenaviruses (GGV, UHV, and Boa AV NL B3) (Table 1A and Fig. 1A, B). Interestingly, the blood of both parental animals in clutch 4 was RT-PCR-positive, but no IB were detected in blood cells. However, the subsequent post mortem analysis of the father revealed IB formation and expression of viral antigen in tissues, confirming BIBD (Table 1).

For clutch 1, comprised of seven embryos in late first trimester (age determined based on the body length of 15 to 17 cm), five embryos were processed for (immuno) histological examination. These did not exhibit IB formation, but were found to weakly express reptarenavirus antigen in occasional cells in brain, liver and kidneys (Fig. 1C). The robust RT-PCR was performed on E1.1 and yielded a positive result (Table 1A). The remaining two embryos (E1.6 and E1.7, Table 1), were used to establish primary cell cultures. These showed viral antigen expression, but no distinct IB formation (Fig. 1D; Table 1).

For clutch 2 (early first trimester embryos with a body length of 5-6 cm), similar results were obtained. Two of the three embryos (E2.1 and E2.2) were used to establish primary cell cultures, which also showed viral antigen expression but no IB. The third embryo (E2.3) was processed for histology and did not exhibit IB but showed occasional weak viral antigen expression in the brain (Table 1A).

Clutch 3 comprised five animals, three of which had been perinatally aborted (PNA3.1 to 3.3). Two of these (PNA3.1 and 3.2) were tested positive for reptarenavirus infection, using the

robust RT-PCR on the brain, and one (PNA3.2) exhibited IB and reptarenavirus antigen in the tissues (Fig. 1E, F). The remaining two animals (J3.1 and 3.2) were euthanized as juveniles two months later. Both were tested positive by the robust RT-PCR on the brain and one also exhibited IB and reptarenavirus antigen in tissues (Table 1A).

The two perinatal abortions of clutch 4 were shown to be infected, using the robust RT-PCR, but did exhibit neither IB formation nor reptarenavirus antigen expression.

Clutch 5 comprised 21 animals. Of the seven juveniles euthanized at the age of eight months, six were diagnosed with BIBD, based on the detection of IB and viral antigen in all examined tissues (Fig. 1G, H), and three of these (3/6) were found positive in the blood by the robust RT-PCR. At the time of euthanasia the samples were collected purely for diagnostic purposes, and unfortunately no samples were stored for RNA isolation. The remaining (1/7) animal (J5.5) was negative in all these tests. Another 11 siblings were euthanized at the age of 12 months. In nine of these, BIBD was confirmed, with the presence of IB and reptarenavirus antigen in tissue and blood cells and a positive result in the robust RT-PCR. Two (2/11) (J5.8, J5.11) were BIBD-negative, but RT-PCR positive in the brain (Table 1A). The last four (4/21) animals were kept by the breeder until they were euthanized at the age of 18 mo (n=2) and 20 mo (n=2) due to the breeder's concern that they suffered from BIBD. These all tested positive for BIBD by histology, IH and robust RT-PCR (Table 1A).

Confirmation of the vertical transmission by next-generation sequencing (NGS)

The primers used for RT-PCR in the preliminary screening were designed for the detection of a subset of reptarenaviruses (GGV, UHV, and Boa AV NL B3) at a time when only four reptarenaviruses were known. However, since we and others [10, 11] thereafter observed that snakes with BIBD are often co-infected with multiple reptarenavirus species, we decided to utilise NGS for further analyses. The NGS study included the first four clutches, but was limited to the animals of which frozen material was available (Table 1A). We removed the reads matching a known snake genome (*Python bivitattus*) from the NGS data and performed

de novo genome assembly. Similarly to our earlier observation [11], several full-length or almost full-length (at maximum some 200-300 nt missing) L and S segments were recovered from the parental samples. In parental animals from breeder 1 (clutches 1 and 3), the following results were obtained: The mother of clutch 1 was positive for six L (Aurora borealis virus-4, ABV-4, GenBank accession KX527594; Tavallinen suomalainen mies virus-1, TSMV-1, KX527595; Hans Kompis virus-1, HKV-1, KX527596; Keijut pohjoismaissa virus-1, KePV-1, KX527597; Bis spöter virus-1, BSV-1, KX527598; Suri Vanera virus, SVaV-2, KX527599) and two S (S6-like, KX527580; S5-like, KX527581) segments, and the mother of clutch 3 was positive for seven L (SVaV-2, KX527587; Kuka mitä häh virus-1, KMHV-1, KX527588; KePV-1, KX527589; University of Helsinki virus-4, UHV-4, KX527590; TSMV-2, KX527591; ABV-4, KX527592; Grüetzi mitenand virus-1, GMV-1, KX527593) and two S (S6-like, KX527578; S5-like, KX527579) segments. Curiously, the brain of the father of clutch 4 was positive for only one pair of L (TSMV-2, KX527582) and S (TSMV-2, KX527575) segments (but several L segments were identified in the serum by RT-PCR, see below), whereas no reptarenavirus genomes were recovered by NGS from the mother despite clear evidence of BIBD (Table 1A). The mother of clutch 2 owned by breeder 2 was positive for four L (ABV-3, KX527583; Kaltenbach virus-1, KaBV-1, KX527584; SVaV-1, KX527585; UHV-3, KX527586) and two S (ABV-2, KX527576; University of Giessen virus-1-like, UGV-1-like, KX527577) segments, whereas no reptarenavirus genomes were recovered from the serum of the father, whose blood cells were also found negative for IB in the cytological examination, providing further evidence that he was indeed not infected at all. The results are summarized in Table 1B and a phylogenetic tree of the *de novo* assembled L and S segments with other reptarenaviruses is shown in Fig. 2A and B. The phylogeny indicates that the arenaviromes of the two snake collections (which never exchanged animals; personal communication) share some common species but also comprise unique viruses.

Initially *de novo* assembly was attempted for several embryos (E1.1, E1.2, E1.7, E2.1 - E2.3), however, this approach was not successful, likely due to inefficient removal of the genomic background during NGS library preparation and low amounts of viral RNA. Instead, we used the reptarenavirus genomes obtained from the parental animal to "fish out" the matching reads from the embryos, an approach we then also took for clutches 3 and 4. However, only scattered reads matching the parental viruses could be recovered from the NGS data for most embryos. Thus we decided to confirm the NGS findings by conventional RT-PCR using virus species-specific (VSS) primers (sequences provided upon request) designed based on the *de novo* assembled arenavirus genomes. We used primers targeting the L segments, since snakes with BIBD most often carry more L than S segments [10, 11]. For most clutches we also included additional samples, from tissues or cell cultures generated from the embryos, into the RT-PCR analysis (Table 1B).

For the three embryos of clutch 1, the "fishing" approach yielded reads matching five (E1.1), two (E1.2) and three (E1.7) of the six L segments and both S segments (all embryos) identified in the mother. For the primary cell culture of E1.7, the reads each covered the entire segments, which might be a consequence of the higher virus content in the supernatant compared to the tissues which were examined for E1.1 and E1.2. The VSS RT-PCRs confirmed the presence of several to all parental viruses in the embryonal tissues (E1.1 and E1.2) and cultured brain cells (E1.6 and E1.7).

For clutch 2, reads matching two L and both S segments were identified by the "fishing" approach from the NGS data of E2.1 (kidney cell culture), E2.2 and E2.3 (both tissue homogenates). The VSS RT-PCRs confirmed the NGS findings and identified the viruses also in homogenates of salpinx and placenta and in cultured cells from umbilicus, placenta and organs (Table 1B).

For clutch 3, we identified reads matching three L and two S segments of the maternal viruses for two perinatal abortions (PNA3.1 and 3.3) and reads matching only two L and two S

segments for the third (PNA3.2), using the "fishing" approach. VSS RT-PCRs on samples from several organs (brain, kidney, liver) confirmed the NGS findings. They also identified maternal viruses in the liver and kidney of the juvenile snakes euthanized at the age of 2 months (Table 1B).

For clutch 4, the "fishing" approach yielded a few reads matching both the L and S segment of the virus identified in the father in one perinatal abortion (PNA4.1), and for the second (PNA4.2), only a single read matching the L segment. Since the subsequent VSS RT-PCR of the PNA samples showed only a weak reaction, we then applied all L segment primers available from the different viruses to RNA extracted from paternal blood and lung, and from the maternal blood sample. Curiously, while the brain of the father remained positive for only a single virus, the blood contained a further 7 reptarenavirus L segments, three of which were also found in the maternal blood. VSS RT-PCRs then identified several paternal viruses in the tissues of both perinatal abortions (Table 1B).

Since the results obtained from clutches 1, 3 and 4 suggested that we had characterized the "virome" of breeder 1´s collection, we did not perform NGS for clutch 5, but tested the father and several of his 12-month-old juvenile offspring, which were in the majority confirmed to suffer from BIBD based on the presence of viral IB and viral antigen in tissues, with all VSS RT-PCRs of the present and an earlier study [11]. The father was positive for four of these viruses, and the juveniles were all found to carry at least two of their father's viruses (Table 1B).

DISCUSSION

So far, studies on the transmission of reptarenaviruses are scarce, and transmission via direct contact, through droplets or aerosols, or via vectors has been discussed [1, 2]. In this study we combined classical and more modern techniques and could show that reptarenaviruses and BIBD can be vertically transmitted. By studying five *Boa constrictor* clutches with BIBD-positive parental animals, we could also demonstrate that several viruses are often co-transmitted vertically from parents to offspring. Using NGS and *de novo* genome assembly we could retrieve nearly complete genomes of several reptarenavirus species for three of the four studied parental snakes. By combining NGS and virus species-specific (VSS) RT-PCRs we could confirm the vertical transmission and show that the offspring retains co-infecting viruses over a long period of time, i.e. for at least 12 months after birth.

In the embryos, infection was not associated with IB formation, but occasional cells in brain, liver and kidneys were found to express viral antigen. Furthermore, primary cell cultures derived from embryos of BIBD positive mothers promoted (part of) the maternal virome and showed viral antigen expression. IB formation was seen in older offspring, first in one of the PNA, consistently in all virus genome-positive juveniles from 2 months of age, confirming that reptarenavirus infection *in vivo* does indeed provoke all the characteristics of BIBD.

Vertical transmission occurs in the reservoir hosts of many arenaviruses. For example, LCMV and MACV can be transmitted transovarially [20, 21] and/or transplacentally [22]. Additionally, infection through semen or maternal blood has been suggested for MACV and Latino virus [23]. Prenatal infection plays an important role in virus maintenance, since for some arenaviruses (LCMV, MACV, and LASV) it may lead to chronic infections [24].

For reptarenaviruses, the precise mode of vertical transmission is not yet known, but our study provides evidence that the viruses of both mother and father can be passed to the offspring, and that the transmission can occur already early in gestation. We were able to isolate viruses also from cell cultures originating from placenta, salpinx, and umbilicus. Since the *Boa constrictor*

embryo does not get into contact with the maternal blood, this indicates that transmission from the mother could also result from contact between maternal tissues and the chorioallantois. However, more detailed studies on the reproductive tract of snakes with BIBD are needed to elucidate the exact mechanisms of transmission from both the maternal and paternal animal.

The convention among snake breeders that also both breeders in our study followed is that the neonates are removed from the mother's cage within a few hours. The clutch is then housed separately until the first shedding at 6-12 days of age, after which the animals are separated and housed in individual cages [25]. This, together with the strict hygiene rules that are applied, does not exclude transmission of viruses between siblings during their first days of life, but renders horizontal infection unlikely thereafter.

It was overall surprising to see how many offspring exhibited reptarenavirus infection without evidence of IB formation or viral antigen expression (4/5 perinatal abortions, one 2-month-old juvenile) or without IB formation and only occasional cells expressing viral antigen (all tested embryos, two 12-month-old juveniles), i.e. BIBD. Also, the fact that we found BIBD-negative animals to carry reptarenaviral RNA in the blood suggests that viraemia may occur frequently, not only in association with the disease, but also in seemingly healthy animals. However, light microscopy and IH are comparatively insensitive methods, and thus the above findings could also be due to low level viral replication. Alternatively, our findings could indicate that reptarenavirus infection has a long incubation period, and both endogenous and exogenous factors could influence the development of BIBD. It has recently been suggested that transient reptarenavirus infections can occur [26]. Although we cannot disprove this assumption, the fact that the vast majority of juvenile offspring from snakes with BIBD in our study had even developed BIBD suggests that at least prenatal reptarenavirus infections generally persist. We recently observed that snakes with BIBD do not often exhibit anti-reptarenavirus antibodies[27]. This could indicate that prenatal infection results in tolerance to reptarenaviruses, allowing

persistent infection. It remains to be determined whether this hypothesis is correct and what determines the subsequent IB formation.

Our observation on the vertical transmission of co-infecting viruses could have direct implications for the evolution of reptarenaviruses. Stenglein et al. recently reported that the co-infection can induce both reassortation and recombination of the viral genomes [10]. Assuming that there is a reservoir host for each reptarenavirus species, it can be hypothesised that, with more relaxed hygiene regimens, housing different snake species in the same facilities has enabled cross-species mixing of the viruses. Vertical transmission of these persistently infecting viruses may have contributed to the plethora of reptarenavirus species that we now detect in captive boid snakes.

In order to avoid infection and/or spreading of the disease within a collection, it would be essential to know all the factors behind reptarenavirus transmission. A six-month quarantine is generally recommended before a new animal is released into a collection, but whether this is sufficient to avoid reptarenavirus transmission is so far unknown [28]. The results that we obtained from clutch 5 indicate that it can take several months before a prenatally infected snake exhibits definite signs of BIBD. In any case, our results demonstrate that animals with BIBD/reptarenavirus infection should not breed, since the likelihood of offspring to become infected is high.

MATERIALS AND METHODS

Ethics statement

All animals included into the study were snakes that were submitted by their owners to the Department of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland. They were euthanized according to a schedule 1 procedure and a full diagnostic post mortem examination was performed. Tissue samples from the dead animals were subjected to the different tests with owners' consent. The owners consented both to the euthanasia and the use of collected samples in this study. Because of suspected BIBD no ethical permissions were required for euthanasia nor the diagnostic-motivated necropsies (both routine veterinary purposes).

Animals and sampling

The study was performed on five *B. constrictor* clutches from two breeders residing in Switzerland. All animals were examined for diagnostic purposes, i.e. BIBD diagnosis, upon the owners' request, which was undertaken on a blood smear and/or by a full post mortem examination. Parental animals that were not euthanised were bled from the tail vein or by cardiac puncture to prepare a blood smear. For necropsy, animals were narcotized with CO_2 followed by decapitation and immediate destruction of the brain by longitudinal sectioning. Immediately after euthanasia, a full post mortem examination performed.

The following *B. constrictor* snakes were examined (Table 1): clutch 1: a BIBD positive (blood smear) pregnant female (euthanized due to emaciation and poor general health) with seven embryos in the first third of gestation; clutch 2: a BIBD positive pregnant female (euthanized due to the owner's suspicion of illness and BIBD) with three embryos in the first third of gestation, the father was subsequently tested on blood smears; clutch 3: three perinatal abortions and two siblings euthanized at the age of two months for diagnostic purposes, blood tested from mother for BIBD diagnosis; clutch 4: two perinatal abortions, blood tested from mother and father for BIBD diagnosis; clutch 5: 22 juveniles (seven euthanized at the age of

eight months, eleven at 12 months, two at 18 months, two at 20 months) for BIBD diagnosis due to positivity of father, post mortem examination of father due to emaciation and chronic pyogranulomatous bacterial rhinitis. The clutch had been separated from the mother within 8 h after birth and individual animals housed separately since after the first shedding at 6-12 days of age.

From all necropsied animals, tissue samples were collected from a range of organs (heart, lung, liver, kidney, and brain), fixed in 10% buffered formalin, and routinely paraffin wax embedded for histological and immunohistological examinations. Selected embryos were fixed and paraffin wax embedded in toto, others were subjected to RNA extraction and/or establishment of cell cultures (Table 1). For adult and juvenile snakes blood smears were prepared and air-dried for cytological examination, and the remaining blood was centrifuged at 1,000 x g for 5 min to separate serum and blood cells. The samples for RNA extraction and/or virus isolation were collected and frozen freshly at -80 °C without fixative or processed immediately.

Cytological, histological, and immunohistological examination

Blood smears were stained with May-Grünwald-Giemsa and a cytological examination was performed to determine the presence of the pathognomonic cytoplasmic IB within blood cells, as previously described [4]. From paraffin blocks, consecutive sections (3-5 µm) were prepared, stained with haematoxylin-eosin (HE) for the identification of the cytoplasmic IB, and subjected to immunohistological staining, using a rabbit anti-UHV NP antibody [14] for the demonstration of reptarenavirus antigen, as described earlier [4].

Cell cultures

From selected embryos (Table 1), samples of brain, heart, liver, kidney, umbilical cord and/or placenta were aseptically collected and subjected to tissue culture (30 °C, 5% CO_2), as described [4]. After passaging of the established cell cultures, aliquots of the cultures (cell-rich

supernatants) were frozen at -80 °C (Table 1) and subsequently used to inoculate permanent boid kidney cell cultures and for virus identification by NGS, and to prepare cell pellets for formalin fixation and paraffin wax embedding, followed by immunohistology for the detection of reptarenavirus antigen, as previously described [4].

Sample preparation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from tissue samples with TRIzol or Trizol LS reagent (Life Technologies) utilizing mechanical homogenization with MagNA Lyser (Roche) following the manufacturer's protocol. From cell culture supernatants (Table 1), RNAs were isolated with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

RNA isolation from blood samples (Table 1) was performed according to a modified protocol for avian blood [29]. Briefly, 100 µl of centrifuged, cell-enriched blood was mixed with 900 µl of TRIzol® (or 250 µl of blood and 750 µl of Trizol LS) and homogenized through pipetting. After addition of chloroform and separation of the RNA containing phase by centrifugation (15 min, 12,000 x g, 4°C) the RNA was purified with the QiaGEN RNeasy Mini Kit (Qiagen) following the manufacturer's protocol for RNA clean up.

The cDNAs were transcribed with random primers using either RevertAid Transcriptase or RevertAid Premium Transcriptase (both from Thermo Fisher Scientific), following the manufacturer's recommendations.

Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) with 1-2 µl of cDNA as the template was utilized in all PCR reactions. Prior to obtaining the NGS data, primers targeting an approximately 170 bp long fragment of the L segment of GGV (1199-1367), UHV (1201-1369), and Boa AV NL3 (1191-1359) were used in PCR amplifications. For Sanger sequencing (performed by the DNA sequencing core facility of Medicum, University of Helsinki, Finland, or by Microsynth, Switzerland), the RT-PCR products were purified using either QIAquick PCR purification Kit (Qiagen) or QIAquick Gel Extraction Kit (Qiagen), both

according to the manufacturer's instructions. After NGS and *de novo* assembly (see below), virus species-specific primers were designed and used for confirming the presence of viruses identified by NGS in parents and the offspring. The primer sequences are provided upon request.

Next-generation sequencing (NGS), de novo assembly, and phylogenetics

The purified RNAs were treated with DNAse I (Fermentas), and re-purified using the GeneJET RNA purification kit (Thermo Fisher Scientific). The RNA was further cleaned using Ribo-Zero Gold rRNA Removal Kit for Epidemiology (Illumina) according to the manufacturer's protocol. The indexed (New England Biolabs) NGS libraries were prepared using the NEBNext Ultra RNA Library Preparation Kit (New England Biolabs) following the manufacturer's instructions. The library quantification was done using NEBNext Library Quant Kit for Illumina (New England Biolabs), and 291-bp paired-end reads of the pooled libraries were sequenced on Illumina MiSeq (Illumina) using MiSeq Reagent Kit v3 (Illumina). Removal of reads matching the host genome and *de novo* sequence assembly were performed using MIRA version 4.0.2. (http://mira-assembler.sourceforge.net/) on CSC (IT Center for Science Ltd., Finland) Taito supercluster. Chipster v.3.1.0. was applied for the generation of subsets and any other handling of the data [30]. The reptarenavirus genomes *de novo* assembled from the parents' samples were used to map the reads matching reptarenaviruses from the offspring samples in Unipro UGENE 1.14.2. [31] utilizing UGENE genome aligner.

Phylogenetic analyses were performed with the newly recovered sequences combined with representative reptarenavirus sequences obtained from the NIAID Virus Pathogen Database and Analysis Resource (ViPR) [32] through the web site at http://www.viprbrc.org/. Complete S segment nt sequences (S6-like clutch 1, KX527580; S5-like clutch 1, KX527581; ABV-2 clutch 2, KX527576; UGV-1 clutch 2, KX527577; S6-like clutch 3, KX527578; S5-like clutch 3, KX527579; TSMV-2 clutch 4, KX527575; ABV-1, KR870010; ABV-2, KR870018; Boa

AV NL3, NC_023761; CASV, NC_018481; GGV, NC_018483; UHV-1, KR870011; UHV-1 (Hetzel et al.), NC_023766; UHV-2, KR870016; UHV-3, KR870019; UGV-1, KR870012; UGV-2, KR870015; UGV-3, KR870013; UGV-4, KR870014; S1, KP071530; S2, KP071541; S3, KP071630; S4, KP071474; S5, KP071599; S6, KP071673; S6A, KP071502; S6B, KP071501; S7, KP071578; S8, KP071509; S9, KP071671; S10, KP071558; S11, KP071559) were aligned with ClustalX [33]. The nt sequences (abbreviation, accession code: ABV-4 clutch 1, KX527594; TSMV-1 clutch 1, KX527595; HKV-1 clutch 1, KX527596; KePV-1 clutch 1, KX527597; BSV-1 clutch 1, KX527598; SVaV-2 clutch 1, KX527599; ABV-3 clutch 2, KX527583; KaBV-1 clutch 2, KX527584; SVaV-1 clutch 2, KX527585; UHV-3 clutch 2, KX527586; SVaV-2 clutch 3, KX527587; KMHV-1 clutch 3, KX527588; KePV-1 clutch 3, KX527589; UHV-4 clutch 3, KX527590; TSMV-2 clutch 3, KX527591; ABV-4-clutch 3, KX527592; GMV-1 clutch 3, KX527593; TSMV-2 clutch 4, KX527582; ABV-1, KR870021; ABV-2, KR870033; ABV-3, KR870025; Boa AV NL3, NC_023762; CAS virus, CASV, NC_018484; Golden Gate virus, GGV, KP071475; HKV-1, KR870028; SVaV-1, KR870024; TSMV-1, KR870026; UHV-1, KR870020; UHV-1 (Hetzel et al.), NC_023765; UHV-2, KR870030; UHV-3, KR870032; UHV-4, KR870027; UGV-1, KR870022; UGV-2, KR870029; UGV-3, KR870023; L1, KP071529; L2, KP071475; L3, KP071523; L4, KP071488; L5, KP071489; L6, KP071492; L7, KP071477; L8, KP071511; L9, KP071563; L10, KP071503; L11, KP071512; L12, KP071550; L13, KP071574; L14, KP071562; L15, KP071551; L16, KP071614; L17, KP071547; L18, KP071481; L19, KP071548; L20, KP071564; L21, KP071478; L22, KP071476) coding for the RNA-dependent RNA polymerase were aligned using amino acid translation guidance in Translator X [34] with the MAFFT algorithm. Phylogenetic trees were reconstructed by the maximum-likelihood method in MEGA 6.06 with 1,000 bootstrap replicates.

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TABLES AND FIGURES

TABLE 1

Clutches, animals and tests performed on individual animals.

A. Summary of results obtained for each animal.

Animals (age)	Cytoplasmic IB/viral antigen			Nucleic acid analysis	
	Blood Cytology	PM Histology	Cell Culture	Robust RT-PCR [specimen]	NGS specimen(s)
Clutch 1, Breeder 1					
Mother	**Pos**	**Pos**[IB, A]		**Pos** [brain]	Brain
E1.1		**Pos**[A]		**Pos** [head]	Head
E1.2		**Pos**[A]		Neg [body]	Body
E1.3		**Pos**[A]			
E1.4		**Pos**[A]			
E1.5		**Pos**[A]			
E1.6			Neg[B]	**Pos** [CCS]	CCS
E1.7			**Pos**[B]	**Pos** [CCS]	CCS
Clutch 2, Breeder 2					
Mother	Neg	**Pos**[IB, A]			Brain
Father[C]	Neg				
E2.1			**Pos**[B]	**Pos** [CCS]	Placenta, CC
E2.2			**Pos**[B]	**Pos** [CCS]	Body, CC
E2.3		**Pos**[A]			Body
Clutch 3, Breeder 1					
Mother[C]	**Pos**			**Pos** [blood]	Serum
PNA3.1		Neg[IB,A]		**Pos** [brain]	Brain
PNA3.2		**Pos**[IB, A]		**Pos** [brain]	Brain
PNA3.3		Neg[IB,A]		Neg [brain]	Brain
J3.1 (2 mo)		**Pos**[IB, A]		**Pos** [brain]	
J3.2 (2 mo)		Neg[IB,A]		**Pos** [brain]	
Clutch 4, Breeder 1					
Mother[C]	Neg			**Pos** [blood]	Serum
Father	Neg	**Pos**[IB, A]		**Pos** [blood]	Serum, lung
PNA4.1		Neg[IB,A]		**Pos** [brain]	Placenta, organs
PNA4.2		Neg[IB,A]		**Pos** [lung]	
Clutch 5, Breeder 1					
Father		**Pos**[IB, A]		**Pos** [brain]	
J5.1 (8 mo)	**Pos**	**Pos**[IB, A]		Neg [blood]	
J5.2 (8 mo)		**Pos**[IB, A]		Neg [blood]	
J5.3 (8 mo)		**Pos**[IB, A]		Neg [blood]	
J5.4 (8 mo)		**Pos**[IB, A]		**Pos** [blood]	
J5.5 (8 mo)		Neg[IB,A]		Neg [blood]	
J5.6 (8 mo)		**Pos**[IB, A]		**Pos** [blood]	
J5.7 (8 mo)		**Pos**[IB, A]		**Pos** [blood]	
J5.8 (12 mo)	Neg	Neg[IB]/(**pos**)[A]		**Pos** [brain]	

J5.9 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.10 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.11 (12 mo)	Neg	Neg^{IB}/(**pos**)^{A}		**Pos** [brain]	
J5.12 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.13 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.14 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.15 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.16 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.17 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.18 (12 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [brain]	
J5.19 (18 mo)		**Pos**$^{IB, A}$		**Pos** [brain]	
J5.20 (18 mo)		**Pos**$^{IB, A}$		**Pos** [brain]	
J5.21 (20 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [blood]	
J5.22 (20 mo)	**Pos**	**Pos**$^{IB, A}$		**Pos** [blood]	

IB - inclusion bodies (as seen in HE stained tissue section or in May Grünwald-Giemsa stained blood smear), PM - post mortem, E - embryo, PNA - perinatal abortion, J - juvenile; CC - cell culture for virus isolation; CCS - supernatant from CC; RT-PCR - reverse transcriptase polymrease chain reaction, NGS - next-generation sequencin;
Pos – positive; (pos) – questionable positive; Neg – negative; blank box – not available/not examined;
A – tested in tissues by immunohistology; B - tested on formalin-fixed, paraffin-embedded tissue culture pellets by immunocytology; C - Animal still alive.

B. Detailed results obtained from the different specimens used for the RT-PCR approach to identify virus species (abbreviations and accession codes are given in materials and methods) in each animal in clutches 1-5.

Animals	Specimen	Viruses detected by virus species-specific (VSS) RT-PCR									
		ABV	SVaV	TSMV	UHV-1/4	L15	L18	L19	L22	HKV	F15-158
Clutch 1, Breeder 1											
Mother	Brain	**ABV**	**SVaV**	**TSMV**		**L15**	**L18**			**HKV**	
E1.1	Head	+	+	+		+	+			+	
E1.2	Body	-	-	+/-		+	+			-	
E1.6	CC (brain)	-	-	+/-		+	-			-	
E1.7	CC (brain)	-	-	+		+	+/-			-	
Clutch 2, Breeder 2											
Mother	Brain	**ABV-3**	**SVaV**		**UHV-1/4**						**F15-158**
Father	Blood	+/-	-		-						-
E2.1	Salpinx	+	+		+						+
	CC (umbilicus)	+	+		-						+
	CC (kidney)	-	-		-						-
	CC (heart)	+	-		-						+/-
E2.2	Body	+/-	+		+						+
	CC (umbilicus)	+	+		+						+
	CC (placenta)	+	+		-						+
	CC (kidney)	+	+		-						+
	CC (liver)	+	+		-						-
E2.3	Body	+/-	+		+						+/-
Clutch 3, Breeder 1											
Mother	Blood	**ABV**	**SVaV**	**TSMV**	**UHV-1/4**	**L15**		**L19**	**L22**		
PNA3.1	Brain	-	-	-	-	-		-	-		
	Kidney	+/-	-	-	-	+		-	-		

	Liver	+	-	-	-	-		+/-	+/-		
PNA3.2	Brain	+	-	-	-	+		-	-		
	Kidney	+	-	-	-	-		+/-	+/-		
	Liver	+	-	-	-	+		+/-	+/-		
PNA3.3	Brain	+/-	-	-	-	+		-	-		
	Kidney	+	-	-	-	+		+/-	-		
	Liver	-	-	-	-	-		+/-	-		
J3.1 (2 mo)	Liver	-	-	+	-	-		+	-		
	Kidney	-	-	+	-	-		+/-	-		
J3.2 (2 mo)	Liver	-	-	-	-	-		-	-		
	Kidney	-	+	+	-	+		+	-		
Clutch 4, Breeder 1											
Father	Blood	**ABV-3**	**SVaV**	**TSMV**	**UHV-1/4**		**L18**	**L19**	**L22**		
	Brain	-	-	+	-		-	-	-		
	Lung	+/-	+	-	+		+	+	+/-		
Mother	Serum	+/-	+	-	-		+	-	-		
PNA4.1	Placenta	+/-	+	+/-	-		+	-	+/-		
	Kidney	+	+/-	+/-	-		+	+	+/-		
	Lung	+	+/-	+/-	+/-		+	-	+/-		
PNA4.2	Placenta	+	-	+/-	-		+	+	+/-		
	Kidney	+	-	+/-	-		+	-	+/-		
	Lung	+	-	+/-	-		+	-	+/-		
	Brain	+/-	+/-	+/-	-		-	-	+/-		
Clutch 5, Breeder 1											
Father	Blood	+ (ABV)	+	+	+	+					
	Liver	+	+	+/-	+/-	+					
J5.8 (12 mo)	Brain	+	-	-	-	+					
J5.8 (12 mo)	Liver	+/-	-	-	-	-					

J5.9 (12 mo)	Liver	+	-	+/-	+	+					
J5.10 (12	Liver	+	+	+	+	+					
J5.11 (12	Liver	+	+	-	+	+					
J5.12 (12	Liver	+	+	+	+	+					
J5.13 (12	Liver	+	+	+	-	+					
J5.14 (12	Liver	+	-	-	-	+					
J5.15 (12	Brain	+	+	+	+	+					
J5.18 (12	Liver	**+**	-	-	-	+					

Bold – L segments derived by NGS and de novo assembly; blank box – not tested

FIGURE 1

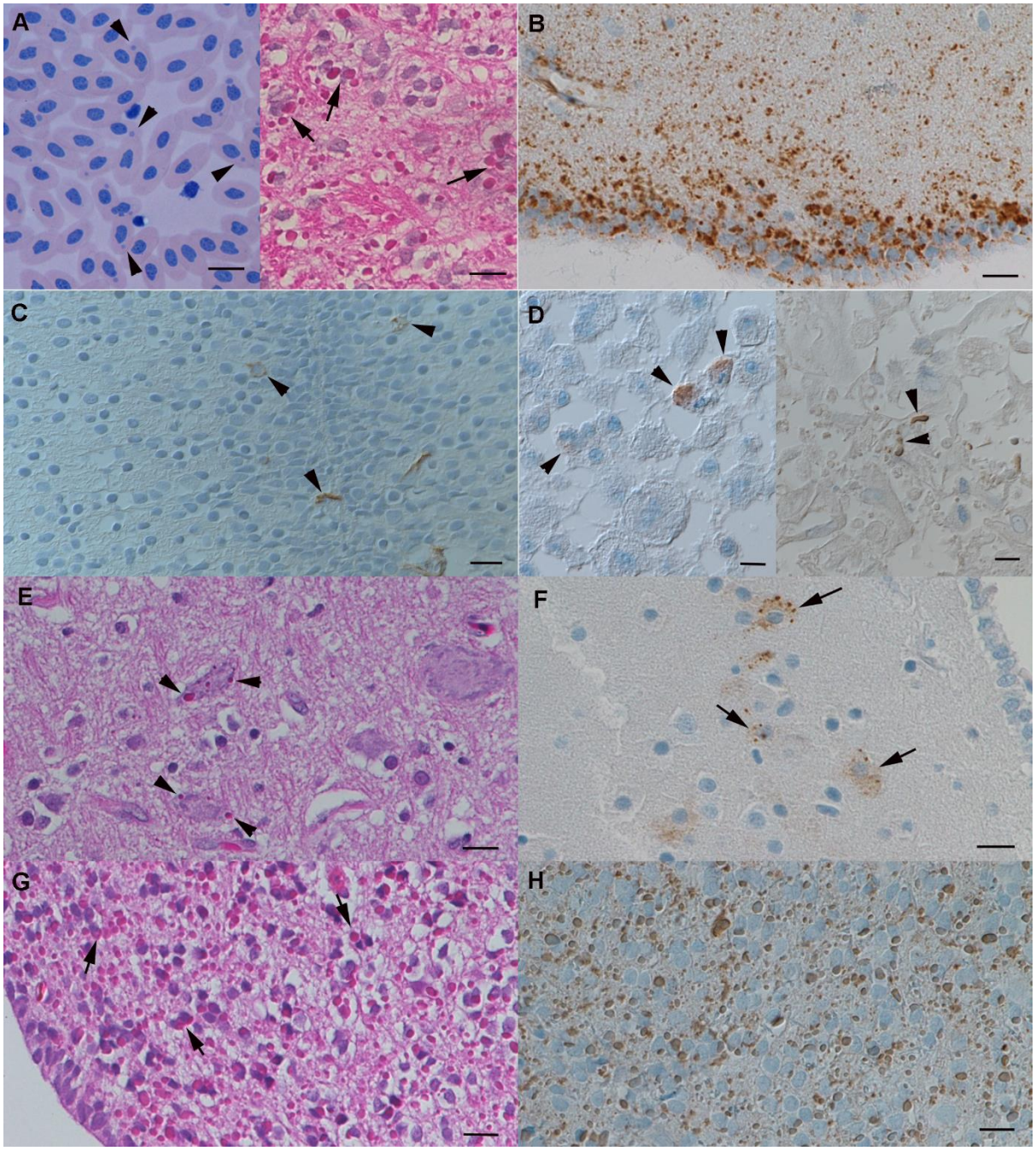

Figure 1. Confirmation of BIBD in parental animals and offspring. **A, B**. Clutch 1, BIBD-positive mother. **A**. The characteristic cytoplasmic inclusion bodies (IB; arrows) are present in erythrocytes (left, blood smear, May-Grünwald Giemsa stain) and in cells in tissues (brain). **B**. Immunohistology confirms the presence of abundant reptarenavirus antigen in all cell types in association with the presence of the IB. **C.** Clutch 2, embryo (E2.1). A few

neurons in the spinal cord exhibit reptarenaviral antigen in the cytoplasm (arrowheads). **D**. Clutch 2, embryo (E2.1). Cell pellet from a brain cell culture. Left: passage 1, right: passage 6. There are individual cells expressing viral antigen (arrowheads). **E, F.** Clutch 3, perinatal abortion (PNA2,2), brain. **E**. A few individual neurons exhibit BIBD IBs (arrows). **F**. Reptarenavirus antigen expression is seen in association with inclusion bodies and dispersed in the cytoplasm (arrows). **G, H**. Clutch 5, juvenile (J5.4), 8 mo, brain. **G**. Abundant IB (arrows) are seen within almost all cells. **H**. Reptarenavirus antigen expression is seen in association with inclusion bodies. A, E, G: haematoxylin eosin stain; B, D, F, H: HRP method, haematoxylin counterstain. Bars = 10 µm.

FIGURE 2

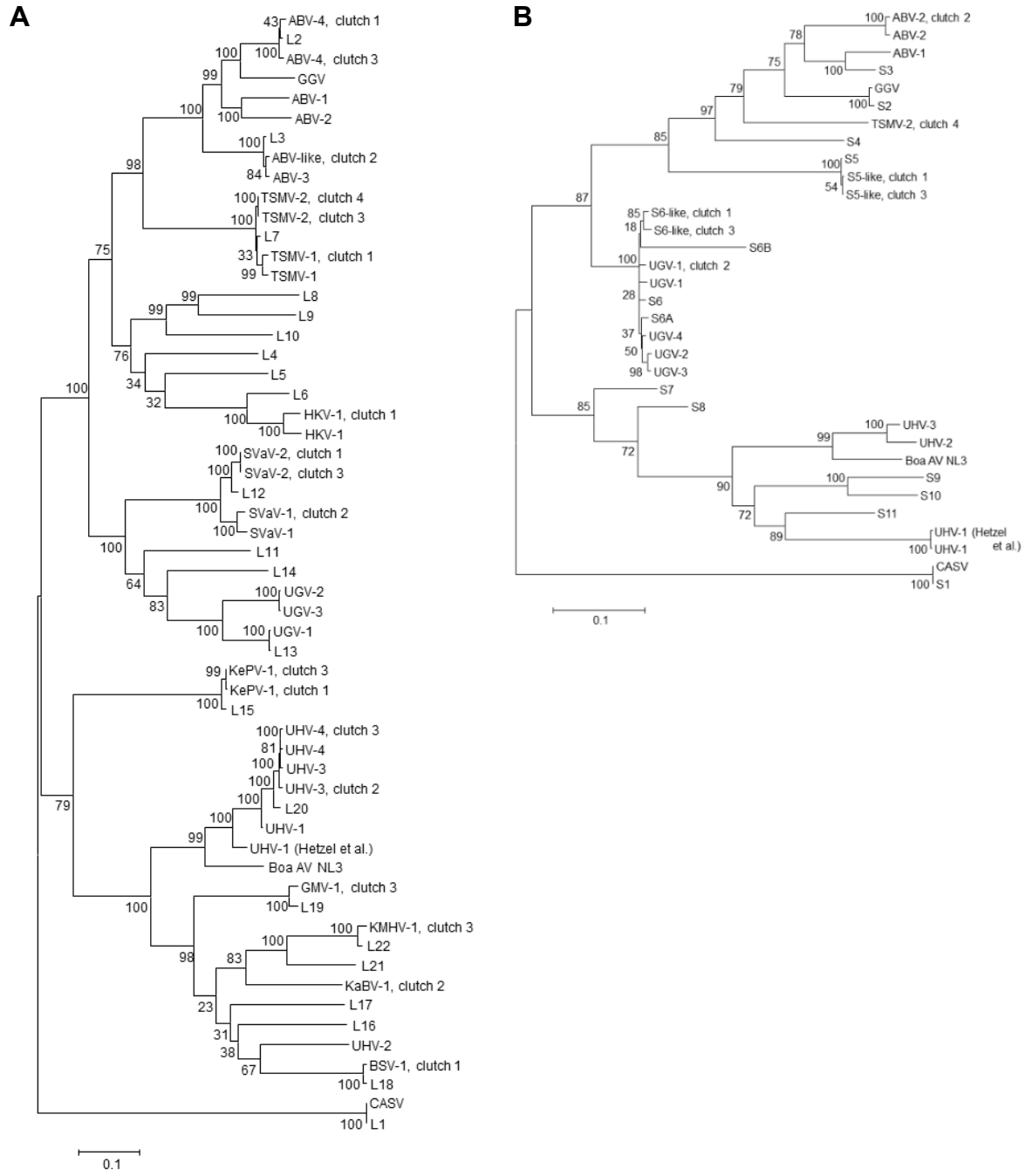

Figure 2. Evolutionary relationships of the reptarenaviruses sequenced in this study. **A**) A Maximum-likelihood tree built on RdRp nt sequences or **B**) complete S segment nt sequences. For simplicity only a single representative of each L or S segment identified by Stenglein et al. is shown. The abbreviations and accession codes are listed in materials and methods. Bootstrap support values of >70 are shown at the nodes.

FIGURE 3

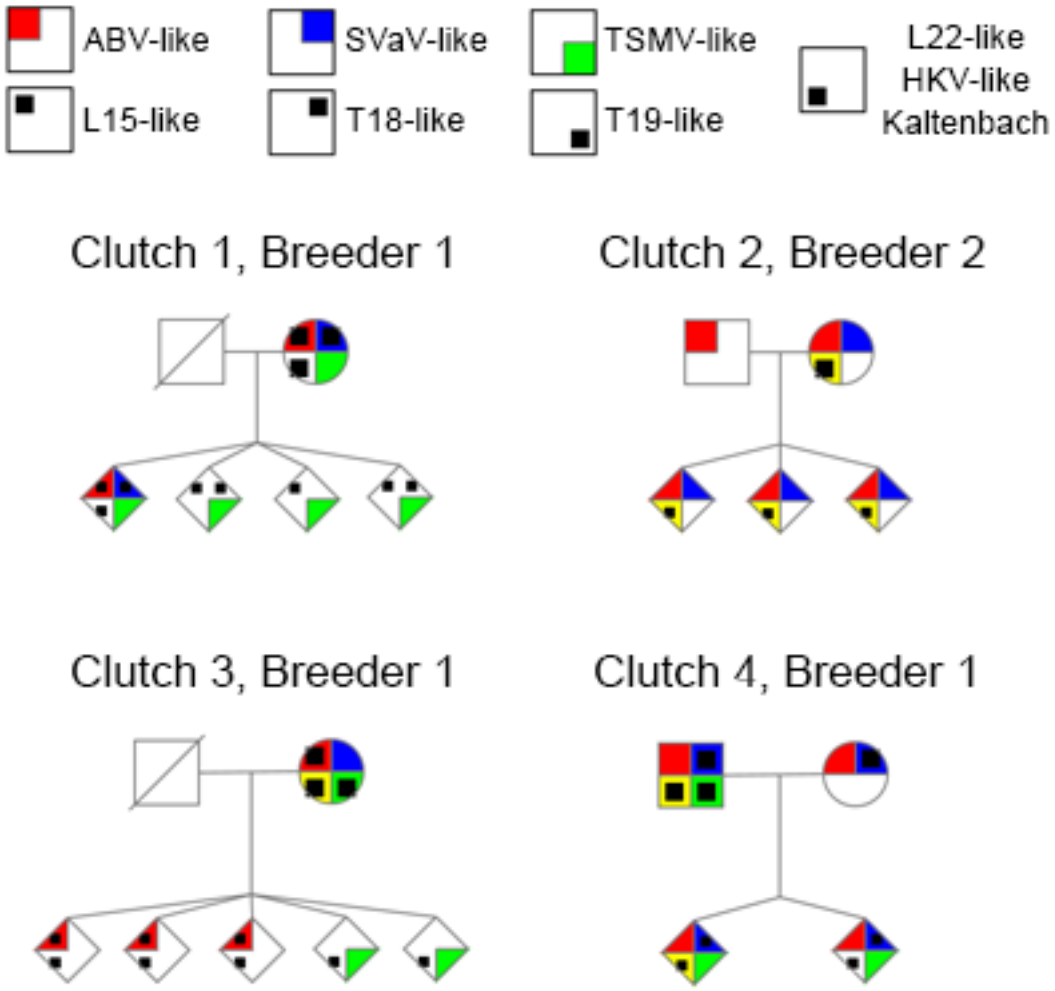

Figure 3. Vertical transmission of viruses presented in the form of a pedigree for the clutches with embryos and perinatal abortions. The viruses sequenced by NGS are indicated by different colours, no samples were available for the fathers of clutches 1 and 3 (indicated by crossed empty box).

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